Brca2 (XRCC11) Deficiency Results in Radioresistant DNA Synthesis and a Higher Frequency of Spontaneous Deletions

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We show here that the radiosensitive Chinese hamster cell mutant (V-C8) of group XRCC11 is defective in the breast cancer susceptibility gene *Brca2*. The very complex phenotype of V-C8 cells is complemented by a single human chromosome 13 providing the *BRCA2* gene, as well as by the murine *Brca2* gene. The Brca2 deficiency in V-C8 cells causes hypersensitivity to various DNA-damaging agents with an extreme sensitivity toward interstrand DNA cross-linking agents. Furthermore, V-C8 cells show radioresistant DNA synthesis after ionizing radiation, suggesting that Brca2 deficiency affects cell cycle checkpoint regulation. In addition, V-C8 cells display tremendous chromosomal instability and a high frequency of abnormal centrosomes. The mutation spectrum at the *hprt* locus showed that the majority of spontaneous mutations in V-C8 cells are deletions, in contrast to wild-type V79 cells. A mechanistic explanation for the genome instability phenotype of Brca2-deficient cells is provided by the observation that the nuclear localization of the central DNA repair protein in homologous recombination, Rad51, is reduced in V-C8 cells.

V-C8 is a Chinese hamster cell mutant that represents the XRCC11 complementation group, among X-ray-sensitive rodent cell mutants, as well as a distinguished group among mitomycin C (MMC)-sensitive rodent cell mutants (for a review, see reference 46). This mutant is extremely sensitive to cross-linking agents, but it also shows an increased sensitivity toward many other DNA-damaging agents, such as methyl methanesulfonate (MMS) and UV light (19, 48). This suggests that the *XRCC11* gene is involved in a wide-ranging cellular response induced by various types of DNA damage. V-C8 cells display radioresistant DNA synthesis (RDS) after ionizing irradiation (36), which is indicative of a defect in DNA damage recognition or cell cycle checkpoint regulation. However, the high level of spontaneous and cross-link-induced chromosomal aberrations manifested by V-C8 cells (19) indicates a possible defect in DNA repair. Indeed, V-C8 cells have an impaired capacity for repair of DNA double-strand breaks (DSBs) after irradiation (36).

In mammalian cells, DSBs are repaired via either nonhomologous end joining (NHEJ) or homologous recombination (HR) (reviewed in reference 14). Genetic complementation studies have determined that V-C8 cells are not defective in DNA-PKcs, Ku80, or Xrcc4, key components of NHEJ, nor in Xrcc2 or Xrcc3, proteins involved in HR, and that this mutant represents a separate complementation group, XRCC11 (36, 46). When compared to cell lines defective in the above-mentioned proteins, the overall phenotype of V-C8 cells more closely resembles those of the Xrcc2- and Xrcc3-defective hamster cell lines irs1 and irs1SF, respectively (8, 12, 16). In common with V-C8, irs1 and irs1SF exhibit an extreme sensitivity to cross-linking agents that is not observed in cell lines defective in NHEJ proteins. This favors the hypothesis that the *XRCC11* gene defective in V-C8 cells might be involved in HR. A key player in DSB repair through HR is the Rad51 protein (reviewed in reference 33). Rad51 is a homolog of the *Escherichia coli* RecA protein. Rad51 facilitates strand invasion of the broken DNA into a homologous double-stranded DNA by forming nucleoprotein filaments. In response to treatment with a number of DNA-damaging agents, Rad51 exhibits a dynamic redistribution within cells, localizing into nuclear foci at sites of DNA damage (28). In mammals, Rad51 belongs to a family of homologous proteins—Rad51, Rad51B, Rad51C, Rad51D, Xrcc2, Xrcc3, and Dmc1—of which the last mainly functions in meiotic cells (reviewed in reference 31). Impaired formation of Rad51 foci, in response to DNA damage, has been demonstrated in hamster or chicken cells defective in the Rad51 paralogs Xrcc2, Xrcc3, Rad51B, Rad51C, and Rad51D, as well as in mammalian Brca1- or Brca2-defective cells (1, 3, 25, 26, 44, 45).

Here we demonstrate that V-C8 is defective in Rad51 foci formation in response to DNA damage, suggesting that the *XRCC11* gene is required for the assembly or stabilization of the Rad51 protein complex. We also show that the phenotype of V-C8 cells is due to a deficiency in Brca2 and that this Brca2

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deficiency leads to RDS after gamma-irradiation. Furthermore, we found that V-C8 cells exhibit a spontaneous mutation spectrum at the *hprt* locus predominated by deletions, unlike wild-type V79 cells. Finally, we demonstrate that the Brca2 protein is required for efficient nuclear localization of the Rad51 protein.

MATERIALS AND METHODS

Cells and culture conditions. The V-C8 mutant cell line derived from Chinese hamster V79 cells has been described previously (19, 36, 48), as has the ataxia telangiectasia (AT)-like mutant cell line V-E5 (47). Cells were routinely cultured in 10-cm dishes (Greiner) in Ham's F-10 (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and PS (100 U of penicillin and 1 mg of streptomycin/ml), without hypoxanthine and thymidine. Monochromosomal MCH204.3 human-mouse hybrid cells, containing human chromosome 13, tagged with a single neomycin resistance gene, were cultured in Dulbecco's modified Eagle medium–F-10 medium, supplemented with 10% FBS and PS, and 800 μg of G418 (Life Technologies)/ml. The simian virus 40-transformed wild-type human fibroblast cell line MRC5V1 (11) was cultured in Dulbecco's modified Eagle medium–F-12 medium supplemented with 10% FBS and PS. Cells were maintained at 37°C in a 5% CO_2 atmosphere humidified to 95 to 100%.

Microcell-mediated chromosome transfer and transfection of exogenous DNA. Introduction of the human chromosome 13 into V-C8 cells by microcell-mediated chromosome transfer was performed basically as described previously (15). As a donor for human chromosome 13, monochromosomal MCH204.3 hybrid cells were used. Microcells were obtained by a 48-h incubation of the MCH204.3 cells with Colcemid (0.05 μg/ml; Life Technologies), followed by 60 min of centrifugation at 34°C (fixed-angle Sorvall GSA rotor, 7,500 rpm, relative centrifugal force value of 5761) in the presence of 20 μg of cytochalasin B (Sigma)/ml. The microcells were filtered in series through 8-, 5-, and 5-μm-pore-size polycarbonate membrane filters (Nuclepore); subsequently added to a monolayer of recipient cells; and finally fused by treatment with 47% polyethylene glycol (Sigma). After 24 h, the cells were cultured under selective pressure of 400 μg of Geneticin (G418 sulfate; Life Technologies)/ml, and microcell hybrid clones were isolated after 10 to 14 days.

Transfections (of the bacterial artificial chromosome [BAC] containing murine *Brca2*, pBAC421-Neo, or of human RAD51-green fluorescent protein [GFP] cDNA) were performed by using the GenePORTER transfection reagent according to the manufacturer's protocol (BIOzym). pBAC421-Neo contains a 220-kb genomic sequence inserted as an *Eco*RI-*Eco*RI fragment in pBACe3.6 (i.e., pBAC108L into which a neomycin resistance gene has been inserted). This insert includes the full-length murine *Brca2* gene. In addition to containing the 60 kb of the gene it contains 70 kb of upstream sequences and 90 kb of downstream sequences. This BAC has been used to completely rescue the embryonic lethality associated with the *Brca2* mutation in mice (S. Swaminathan and S. K. Sharan, unpublished data).

Clonogenic survival assays. Cell cultures in exponential growth were trypsinized, and 300 to 700 cells (3,000 to 28,000 cells for the highest doses) were seeded into 10-cm dishes (two dishes per dose; three dishes for the untreated control) and left to attach for 4 h. The cells were irradiated with X rays (dose rate, ~2.8 Gy/min; 200 kV; 4 mA; 0.78 mm Al), treated with MMC (continuous), or treated with MMS (1 h). After MMS treatment, the cells were rinsed twice with phosphate-buffered saline (PBS) and fresh medium was added. After 8 to 12 days, the cells were rinsed with 0.9% NaCl and stained with 0.25% methylene blue, and visible colonies were counted. Each survival curve represents the mean of at least three independent experiments. Error bars represent the standard errors of the mean.

Immunofluorescence labeling and microscopy. To examine centrosomes or Rad51 focus formation, cells were seeded 1 day prior to analysis onto glass slides, giving subconfluent cells at time of fixation. For centrosome analysis, the cells were fixed with methanol-acetone (7:3). For Rad51 focus analysis, cells were either mock treated or else irradiated with 12 Gy of X rays (dose rate, ~2.8 Gy/min; 200 kV; 4 mA; 0.78 mm Al) or incubated with 2.4 μg of MMC/ml for 1 h, after which the cells were washed with PBS (10 mM Na_2HPO_4, 0.14 mM NaCl; pH 7.4), and fresh medium was given. Eight hours after (mock) treatment, the cells on the slides were fixed with 2% paraformaldehyde.

After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS and blocked with PBS$^+$ (20 mM glycine [J. T. Baker]–0.5% [wt/vol] BSA [Sigma] in PBS). The cells were incubated with rabbit antiserum against γ-tubulin (Sigma) for centrosome analysis or against human Rad51 (FBE-2) and subsequently with

Alexa 488-conjugated goat anti-rabbit immunoglobulin G (IgG) (Molecular Probes). Cell nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole; Sigma). Centrosomes and Rad51 foci were examined under a Leitz Axioplan fluorescence microscope.

Measurement of RDS after gamma-irradiation. Measurement of RDS was performed essentially as described but without the trichloroacetic acid precipitation step (47). In short, cells were seeded in duplicate 30-mm dishes (four for the unirradiated control) and were prelabeled overnight with [^{14}C]thymidine (Amersham) in HEPES-buffered thymidine-free Ham's F-10 medium and then exposed to graded doses of ^{137}Cs gamma-irradiation (1.2 Gy/min) and subsequently labeled with [^{3}H]thymidine (Amersham) for 4 h. Free thymidine pools were chased by a further 30- to 45-min incubation in unlabeled medium. Scintillation-counted ^{3}H/^{14}C radioactivity ratios of alkali-lysed cells were taken as a measure of DNA synthesis rates and plotted as percentages of unirradiated cells.

Analysis of chromosomal aberrations and SCEs. Frequencies of spontaneous or MMC-induced chromosomal aberrations or sister chromatid exchanges (SCEs) were determined in exponentially growing cell cultures. The cells were either mock treated or treated for 24 h (chromosomal aberrations) or 2 h (SCEs) with 0.25, 0.5, and 1 ng of MMC/ml (V-C8) or with 1, 15, 30, 40, or 80 ng of MMC/ml (V79 and V-C8 with human chromosome 13 or the BAC containing murine *Brca2*). For SCE analysis, 5-bromo-2′-deoxyuridine (Sigma) was then added to the medium (10 μM final concentration) to enable sister chromatid differentiation. The cells were harvested by trypsinization 24 to 28 h after (mock) treatment, after 2 h of incubation with 1 μg of Colcemid/ml. The cells were fixed, after treatment with hypotonic solution (0.6% sodium citrate), in ethanol-glacial acetic acid (3:1). Air-dried preparations were made and stained with Giemsa or with fluorochrome plus Giemsa. For chromosomal aberrations, 100 mitotic cells were analyzed at each dose, and SCEs were scored in 25 cells. All experiments were repeated at least once.

Immunoblot analysis. For immunoblot analysis, whole-cell extracts were made by resuspending cell pellets in lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholic acid, 1 mM EDTA [pH 7.4], 1 mM dithiothreitol, 0.5 mg of Pefabloc/ml, 1 μg of aprotinin/ml, 1 μg of leupeptin/ml), followed by sequential snap-freezing on dry ice and thawing at 30°C three times. Cell debris was removed by centrifugation at 14,000 rpm at 4°C for 15 min. For determination of the subcellular localization of Rad51-GFP, cellular fractionation was performed as described previously (4). The quality of the fractions was checked by using a monoclonal antibody against the nuclear protein p62 (6). Protein concentrations were determined by using the Bradford protein assay with bovine serum albumin as the standard. Protein (100 μg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Bio-Rad), and probed with polyclonal antiserum against human Rad51 (FBE-2) or mouse Brca2 (pep-4) or with monoclonal antiserum against human BRCA2 (Ab-1; Oncogene) or GFP (Roche). The membranes were then probed with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG, and antibody binding was detected by enhanced chemiluminescence (Amersham). The equality of loading was confirmed by probing with monoclonal antiserum against actin (Santa Cruz).

Determination of the spontaneous mutation spectrum at the *hprt* locus. For the isolation of spontaneous *hprt* mutants, V-C8 cells were divided among 25 independent cultures of 10^3 cells per dish. Each population was propagated separately to ensure that all *hprt* mutants obtained were independent. From each population of ca. 3×10^7 cells, 2×10^6 cells were plated in 6-thioguanine-containing medium (5 μg/ml) at a density of 10^5 cells per dish. After 10 days, only one 6-thioguanine-resistant colony was isolated per culture. In all, 21 independent spontaneous *hprt* mutants were isolated. PCR analysis of *hprt* cDNA and genomic DNA, as well as sequence analysis of *hprt* cDNA, was performed as described earlier (30).

RESULTS

Impaired Rad51 focus formation in V-C8 cells. To investigate whether V-C8 cells are defective in HR, we examined the ability of these cells to form Rad51 foci after DNA damage induction. V-C8 cells were treated with X rays or MMC. After 8 h, the cells were fixed, and Rad51 foci were visualized microscopically by labeling the cells with rabbit polyclonal antiserum against Rad51, followed by labeling with a rabbit specific fluorophore-conjugated antibody. We found that V-C8 cells were clearly defective in Rad51 focus formation after X-ray or MMC treatment (Fig. 1). While treatment with X rays
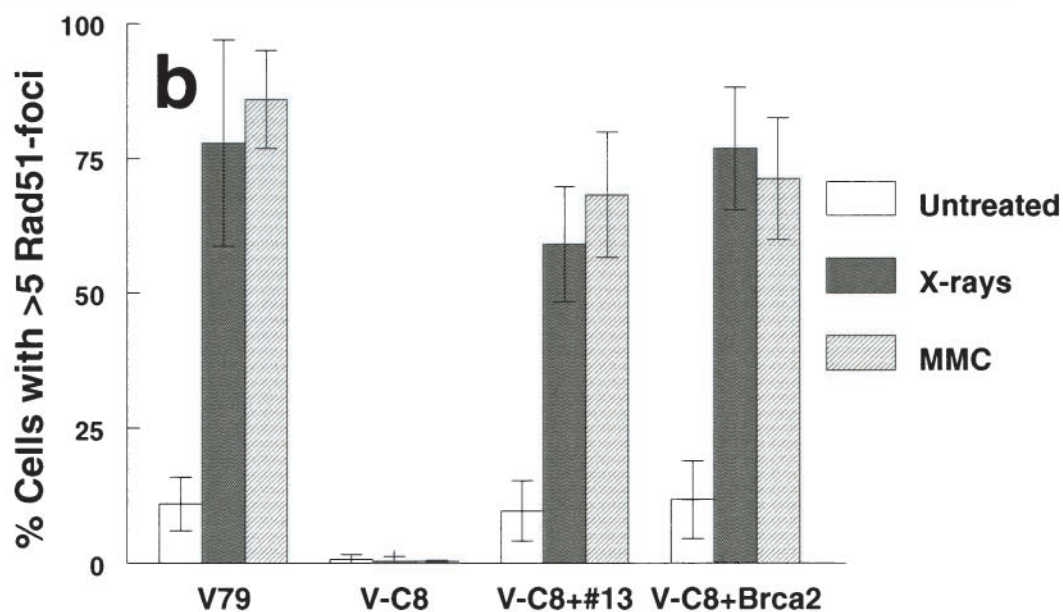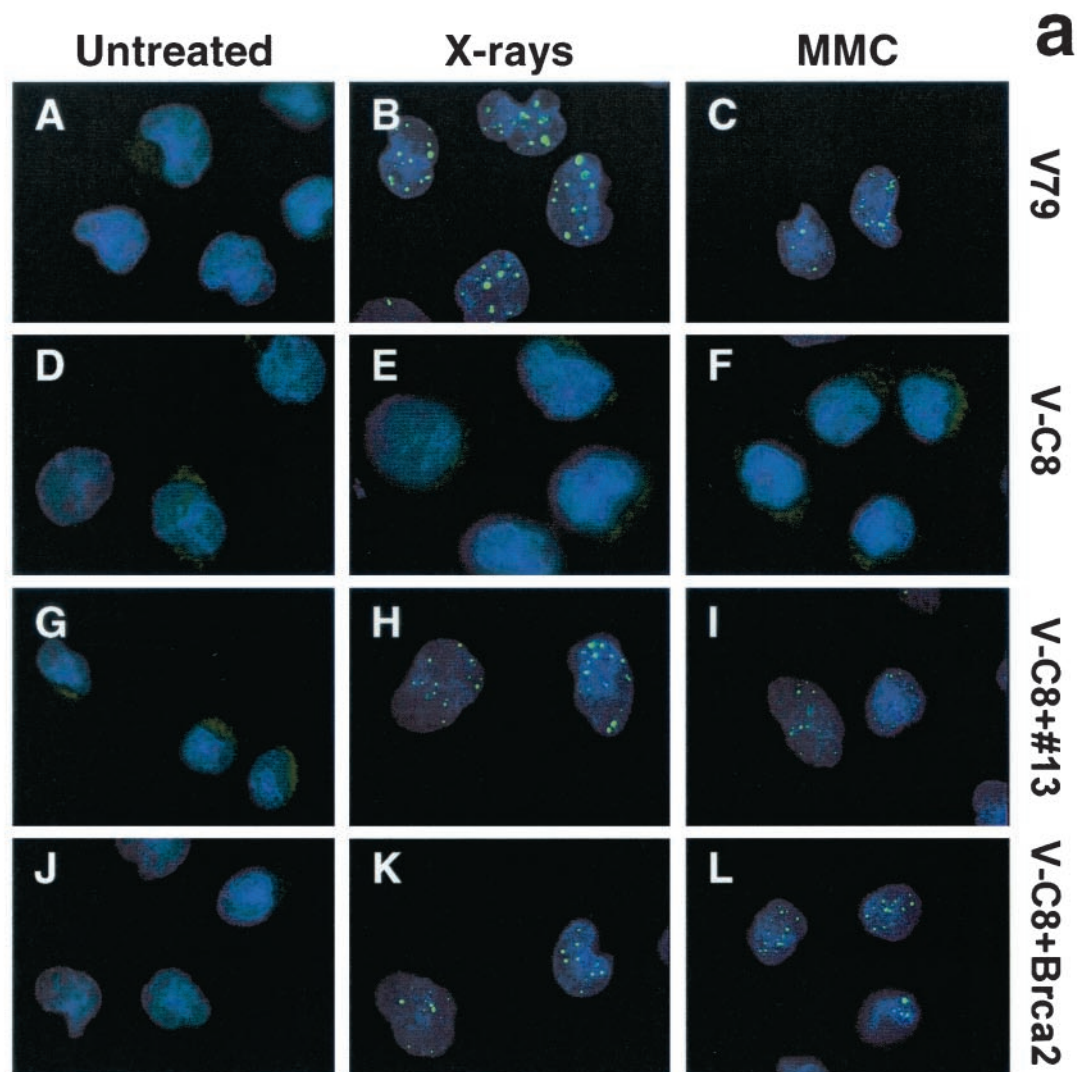
FIG. 1. (a) Immunofluorescent visualization of Rad51 nuclear foci: wild-type V79 (A to C), V-C8 (D to F), V-C8 cells with human chromosome 13 providing the *BRCA2* gene (V-C8+#13) (G to I), and V-C8 containing a BAC with the murine *Brca2* gene (V-C8+Brca2) (J to L). Cells were analyzed for 8 h after treatment with either 12 Gy of X rays (B, E, H, and K) or 2.4 µg of MMC/ml for 1 h (C, F, I, and L). (b) Quantification of Rad51 focus-positive cells. A cell containing more than five distinct foci was considered positive. Each bar represents the result of scoring at least 100 cells. The error bars represent the standard error of the mean (SEM).
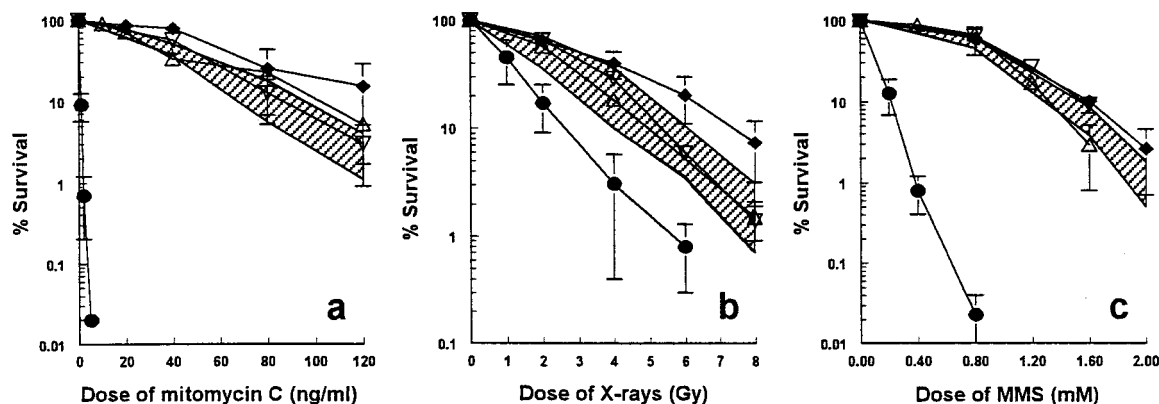
FIG. 2. Cell survival after exposure to MMC (a), X rays (b), or MMS (c) of wild-type V79 (♦), V-C8 (●), V-C8 cells with human chromosome 13 providing the *BRCA2* gene (V-C8+#13) (shaded area, four independent clones), and V-C8 containing a BAC with the murine *Brca2* gene (V-C8+Brca2, two independent clones; △ and ▽). The error bars represent the SEM.

or MMC induced Rad51 foci in ~80% of wild-type V79 cells after 8 h, in V-C8 cells the percentage of cells positive for Rad51 foci remained at a background level. In untreated cells it has been found that Rad51 foci form typically in S phase (27). The lack of detection of Rad51 foci in V-C8 cells cannot be due to differences in cell cycle distribution, especially in S phase, because this was found to be similar in V79 and V-C8 cells after treatment with MMC or X-ray irradiation (20; unpublished results). Immunoblot analysis of whole-cell extracts of V-C8 cells showed normal levels of Rad51 protein (data not shown). Taken together, these results suggest that the *XRCC11* gene is involved in the assembly and/or stabilization of the Rad51 protein complex.

Complementation of cross-sensitivity and Rad51 focus formation by human chromosome 13 and by a BAC containing the murine *BRCA2* gene. To identify the defective gene in V-C8 cells, we examined candidate genes involved in Rad51 complex assembly for their ability to complement the defects of these cells. Transfection of *hRad51* cDNA, as well as transfer of a single human chromosome 15 (providing *hRAD51*), did not complement the MMC sensitivity of V-C8 cells. Furthermore, sequencing of V-C8 *Rad51* cDNA revealed no mutations (data not shown). No complementation was found after transfection of V-C8 cells with cDNA of the Rad51 paralogs *hRAD51B*, *hRAD51C*, and *hRAD51D* (data not shown). As mentioned in the introduction, it had already been determined by previous genetic complementation studies that V-C8 cells are not defective in *Xrcc2* or *Xrcc3* (46). Therefore, the *Brca1* and *Brca2* genes were considered as candidate defective genes.

To examine the effect of the *BRCA2* gene on V-C8 cells, a single human chromosome 13 providing this gene (39) was transferred into these cells by microcell-mediated chromosome transfer. Several V-C8+#13 microcell hybrid clones were obtained and examined for complementation of the defect in V-C8 cells. The sensitivities of V-C8 to MMC, X rays, MMS (Fig. 2), and UV light (data not shown) were largely complemented by human chromosome 13. Transfer of human chromosome 17 providing the *BRCA1* gene, as well as transfer of several other single human chromosomes (8, 9, 11, 12, 18, 19, 21), did not result in any complementation of V-C8 cells (data not shown). Taken together, these results indicate that only

human chromosome 13 complements the defect in V-C8 cells, suggesting that the *BRCA2* gene might be responsible. To confirm this, we transfected V-C8 cells with a BAC containing the murine *Brca2* gene and found complementation of clonogenic survival, similar to that observed with V-C8+#13 microcell hybrids (Fig. 2). Both the human chromosome 13 and the murine *Brca2* gene restored the ability of Rad51 focus formation in response to X-ray irradiation or treatment with MMC (Fig. 1).

Complementation of RDS. V-C8 cells display RDS (36), a phenomenon characteristic of cells derived from patients with AT and the AT-like hamster mutant cell lines of group XRCC8 (13, 32, 47). To investigate whether the RDS phenotype seen in V-C8 cells is due to a defective Brca2 protein, we examined whether the *BRCA2* gene restored DNA synthesis inhibition in this cell line after ionizing irradiation. Data presented in Fig. 3 show that the dose response of DNA synthesis following gamma-irradiation was restored by the addition of a single human chromosome 13, providing the *BRCA2* gene, suggesting that the RDS phenotype of V-C8 cells is due to a defect in Brca2.

Complementation of spontaneous and MMC-induced chromosomal aberrations. V-C8 cells display tremendous genomic instability (Tables 1 and 2) (19). V-C8 cells show ca. 25-fold more spontaneous aberrations than wild-type V79 cells. The majority of spontaneous chromosomal exchanges in V-C8 were of the chromatid type (Table 1), which are usually lethal. In V79 these were rarely found. After treatment with MMC, the relative chromosomal sensitivity of V-C8 was increased ca. 600-fold compared to V79 cells (Table 2). Cells in which the human or murine *Brca2* gene was expressed had clearly reduced levels of spontaneous and MMC-induced chromosomal aberrations, indicating that defective Brca2 in V-C8 cells is responsible.

Expression of Brca2 in V-C8 cells. The data presented above show that the *Brca2* gene complements the cross-sensitivity to DNA-damaging agents, Rad51 focus formation, RDS, and chromosomal aberrations. We conclude therefore that V-C8 is defective in Brca2. To verify this conclusion, the expression of *Brca2* was examined in V-C8 cells by immunoblot analysis. Significantly, Brca2 protein was not detected in V-C8 cells,
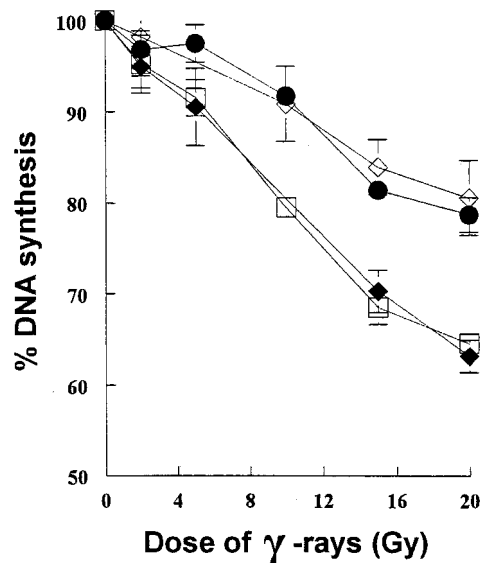
FIG. 3. Dose-response curve of the rate of DNA synthesis after gamma-irradiation in V-C8 cells with human chromosome 13 providing the *BRCA2* gene. The curves shown are for wild-type V79 cells (◆), V-C8 cells (●), V-C8+#13 (□, two independent clones), and the AT-like hamster cell line V-E5 (◇). The error bars represent the SEM.

whereas V79 cells clearly expressed Brca2 (Fig. 4a). The antibody used, pep-4, has been raised against the 13 most carboxy-terminal amino acids of the mouse Brca2 protein. These results suggest that in V-C8 cells Brca2 is not expressed, is truncated, or is unstable. The data presented in Fig. 4b show that in V-C8 cells complemented by a single human chromosome 13 (clones #13-5 and #13-10), the BRCA2 protein could be detected, as in wild-type human MRC5V1 cells, whereas clone #13-7, which is not complemented for focus formation and clonogenic survival, does not express the BRCA2 protein. The antibody used for this immunoblot, Ab-1, has been raised against amino acids 1651 to 1821 of human BRCA2 and does not recognize the hamster protein (Fig. 4b, lanes 1 and 2). Equality of loading was confirmed by probing with monoclonal antiserum against actin (not shown). Taken together, these results strengthen our conclusion that the phenotype of V-C8 cells is indeed due to defective Brca2.

TABLE 2. Relative sensitivities for chromosomal aberrations[a]

Cell line	Relative sensitivity			
	V79	V-C8	V-C8+#13	V-C8+Brca2
Spontaneous	1[b]	26	5	6
MMC induced	1[c]	635	3	11

[a] Relative sensitivities were calculated from the slopes of the dose-response curves for MMC-induced chromosomal aberrations.
[b] The frequency of spontaneous aberrations per 100 cells in wild type V79 was 7 ± 2, and this was set at 1.
[c] The slope of the induction curve of chromosomal aberrations after MMC treatment in wild-type V79 cells was 0.4 aberration per 100 cells per ng of MMC/ml, and this was set at 1.

Spontaneous and MMC-induced SCEs. Chicken cells defective in one of the paralogs of Rad51 all show reduced frequencies of both spontaneous and MMC-induced SCEs (23, 25, 26). To determine whether Brca2 deficiency also leads to reduced levels of SCEs, the frequency of SCEs was measured in V-C8 cells. The number of spontaneous SCEs per cell in V-C8 was found to be similar to that in V79 cells (Table 3). In contrast, after treatment with different doses of MMC, a clear induction of SCE levels with dose was seen in V79 cells (15.0 ± 0.7 at 15 ng/ml and 21.3 ± 1.3 at 30 ng/ml) but not in V-C8 of cells. Due to the MMC toxicity for V-C8 cells, measurable metaphase spreads could be obtained only with doses of up to 0.5 ng of MMC/ml. With these low doses, however, SCE levels were found to be similar to the spontaneous level (Table 3). Interestingly, in V-C8 cells with an additional human or mouse *Brca2* gene, SCEs were induced after MMC treatment, although at a lower frequency than in V79 cells. These results suggest that V-C8 cells are defective in MMC-induced SCE formation and that the human or mouse *Brca2* gene partially complements this defect. Therefore, Brca2 might play a role in the formation of SCEs in the normal cellular response to cross-links.

Abnormal centrosomes in V-C8 cells. Besides defective HR, chromosomal instability can also be caused by incorrect segregation of chromosomes into daughter nuclei during cell division. Chromosome segregation is mediated by the spindle poles, which nucleate from the centrosome. The centrosome normally duplicates once per cell cycle, at the G_1-S boundary, and the two daughter centrosomes migrate to opposite poles prior to chromosome segregation (50). Brca2-defective mouse

TABLE 1. Spontaneous and MMC-induced chromosomal aberrations per 100 cells

Cell line	Treatment[a] (ng/ml)	Chromatid		Chromosome		Total no. of breaks[c] ± SEM
		No. of breaks	No. of exchanges[b]	No. of breaks	No. of exchanges	
V79	Spontaneous	6	0	1	0	7 ± 2
	MMC (80)	26	4	7	1	43 ± 7
V-C8	Spontaneous	134	12	19	3	183 ± 20
	MMC (0.5)	241	29	5	4	312 ± 39
V-C8+#13	Spontaneous	29	0	5	0	34 ± 4
	MMC (80)	75	14	10	1	115 ± 4
V-C8+Brca2	Spontaneous	32	1	5	1	41 ± 4
	MMC (80)	277	58	9	1	404 ± 135

[a] The depicted frequencies of MMC-induced chromosomal aberrations are after treatment with equitoxic doses, which give 30 to 40% survival.
[b] Chromatid exchanges are interchanges, intrachanges, triradials, or subchromatid exchanges.
[c] For calculations of total breaks, the exchanges were counted as two breaks.
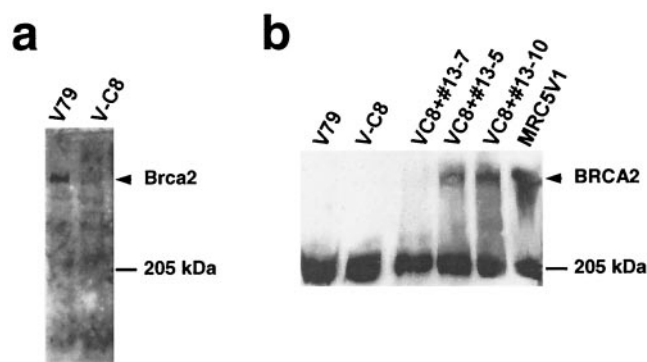
FIG. 4. Immunoblot analysis of the hamster Brca2 protein in V-C8 and V79 cells with antibody pep-4 raised against the 13 most carboxy-terminal amino acids of the mouse Brca2 protein (a) and the human BRCA2 protein in V-C8+#13 microcell hybrids with antibody Ab-1, raised against amino acids 1651 to 1821 of human BRCA2 (b). The wild-type simian virus 40-transformed human fibroblast cell line MRC5V1 was used as a positive control.

embryonic fibroblasts (MEFs) show abnormal centrosome duplication, resulting in multiple centrosomes per cell (34). We investigated whether V-C8 cells displayed aberrant centrosome duplication as well (Fig. 5). Centrosomes were visualized microscopically by labeling the cells with rabbit antiserum against γ-tubulin, followed by labeling with a rabbit specific fluorophore-conjugated antibody. The data presented in Fig. 5 show that in 89% of V79 cells the centrosomes were visible as clearly defined, dot-shaped structures, while in the majority of V-C8 cells (64%) the γ-tubulin staining was scattered and diffuse. This was partially complemented by human chromosome 13 and the murine *Brca2* gene. These results suggest that Brca2 deficiency leads to the formation of aberrant centrosomes. In order to find out whether this scattered γ-tubulin staining could represent functional multiple centrosomes, we labeled V-C8 cells with β-tubulin to visualize the spindles in mitotic cells. We found that only 1 of 18 mitotic V-C8 cells examined with scattered γ-tubulin staining showed a multipolar spindle (data not shown). This suggests that, despite the aberrant centrosome structures, bipolar cell division still takes place in most V-C8 cells.

Molecular analysis of *hprt* mutations in V-C8. To investigate the molecular nature of *hprt* mutations in V-C8, 21 independent spontaneously arisen *hprt* mutants were isolated. From every mutant clone, cytoplasmic RNA was isolated, allowing *hprt* cDNA synthesis, amplification of the *hprt* coding region,

and direct sequencing of the PCR-amplified cDNA. In 10 of the cases, no detectable amounts of *hprt* cDNA could be obtained after PCR. Therefore, genomic DNA was isolated and used for PCR with exon-specific primers. The nature of the molecular changes of the spontaneous *hprt* mutants of V-C8 is shown in Table 4. The mutation spectrum is predominated by deletions (11 mutants; 52%), which are mainly large deletions of one or more exons, while base substitutions were found in only two mutants (10%). This mutation spectrum significantly differs from that of V79 cells (49). In V79 cells, mainly base substitutions have been found (43% transversions, 21% transitions), while deletions were found in only 13% of the *hprt* mutants. This suggests that the Brca2 protein is involved in a specific process that prevents the formation of deletions rather than protecting against mutations in general.

Subcellular localization of Rad51. To gain insight into the underlying reason for the failure to detect efficient DNA damage-induced Rad51 focus formation in V-C8 cells, we investigated Rad51 focus formation in living cells. To this end, stable transfectants of parental wild-type hamster V79 and the Brca2-deficient V-C8 derivative, expressing a fusion between Rad51 and GFP, were generated. Upon treatment of the cells with ionizing radiation, Rad51-GFP accumulated into nuclear foci in V79 cells but not in V-C8 cells (Fig. 6a), just as was observed for the endogenous Rad51 protein (Fig. 1). Interestingly, although the immunofluorescence protocol for the detection of endogenous Rad51 did not detect the presence of Rad51 in the cytoplasm (Fig. 1) (10), the majority of Rad51-GFP was located there. Immunoblot analysis of nuclear and cytoplasmic fractions of the Rad51-GFP-expressing cells confirmed that the majority of the Rad51-GFP was located in the cytoplasm, in both V79 and V-C8 cells (Fig. 6b). In addition, the level of Rad51-GFP present in the nucleus was reduced in V-C8 cells compared to V79 cells (Fig. 6b). Immunoblotting with an anti-Rad51 antibody showed that the endogenous and GFP-tagged variant of Rad51 behaved similarly. Our results are in agreement with a recent study that demonstrates the presence of Rad51 in the cytoplasm and shows a reduction in the nuclear protein level of RAD51 in BRCA2-deficient human CAPAN-1 cells (4). The failure to detect efficient DNA damage-induced Rad51 focus formation in Brca2-deficient cells might be related to a reduction in the level of Rad51 that is present in the nucleus.

DISCUSSION

We have found that the Chinese hamster cell mutant, V-C8, of group XRCC11 is defective in Rad51 focus formation after DNA damage. This, as well as cross-sensitivity to DNA-damaging agents, RDS, and chromosomal aberrations, could be rescued by a functional *Brca2* gene, suggesting that Brca2 is defective in these cells. This was supported further by immunoblot analysis which showed that the expression of Brca2 was severely reduced or absent in V-C8 cells, indicating that the phenotype of V-C8 cells is indeed due to a deficiency in Brca2.

The human *BRCA2* gene has been identified as a tumor suppressor gene (29), of which germ line mutations are responsible for about one-third of the familial breast cancer cases (7). The BRCA2 protein has been found to play an important role in cellular responses to DNA damage and in maintaining

TABLE 3. Frequencies of spontaneous and MMC-induced SCEs

Dose of MMC (ng/ml)	No. of SCEs per cell ± SD			
	V79	V-C8	V-C8+#13	V-C8+Brca2
0	6.6 ± 0.4	5.4 ± 0.4	6.3 ± 0.4	5.5 ± 0.3
0.25	ND[a]	4.8 ± 0.4	ND	ND
0.5	ND	6.9 ± 0.5	ND	ND
1	5.9 ± 0.4	–	ND	ND
5	ND	–	6.9 ± 0.4	6.5 ± 0.3
15	15.0 ± 0.7	–	8.4 ± 0.5	8.5 ± 0.5
30	21.3 ± 1.3	–	–	–

[a] ND, not determined; –, not measurable.

a

| Cell line | No. of cells | Centrosomes[a] | |
		Normal	Aberrant
V79	207	184	23 (11%)
V-C8	228	83	145 (64%)
V-C8+#13	403	256	147 (36%)
V-C8+Brca2	261	137	124 (48%)

[a] aberrant: scattered. diffuse γ-tubulin staining like in Fig. 5b. right panel (normal: clearly defined dot-shaped γ-tubulin staining like in Fig. 5b. left panel).
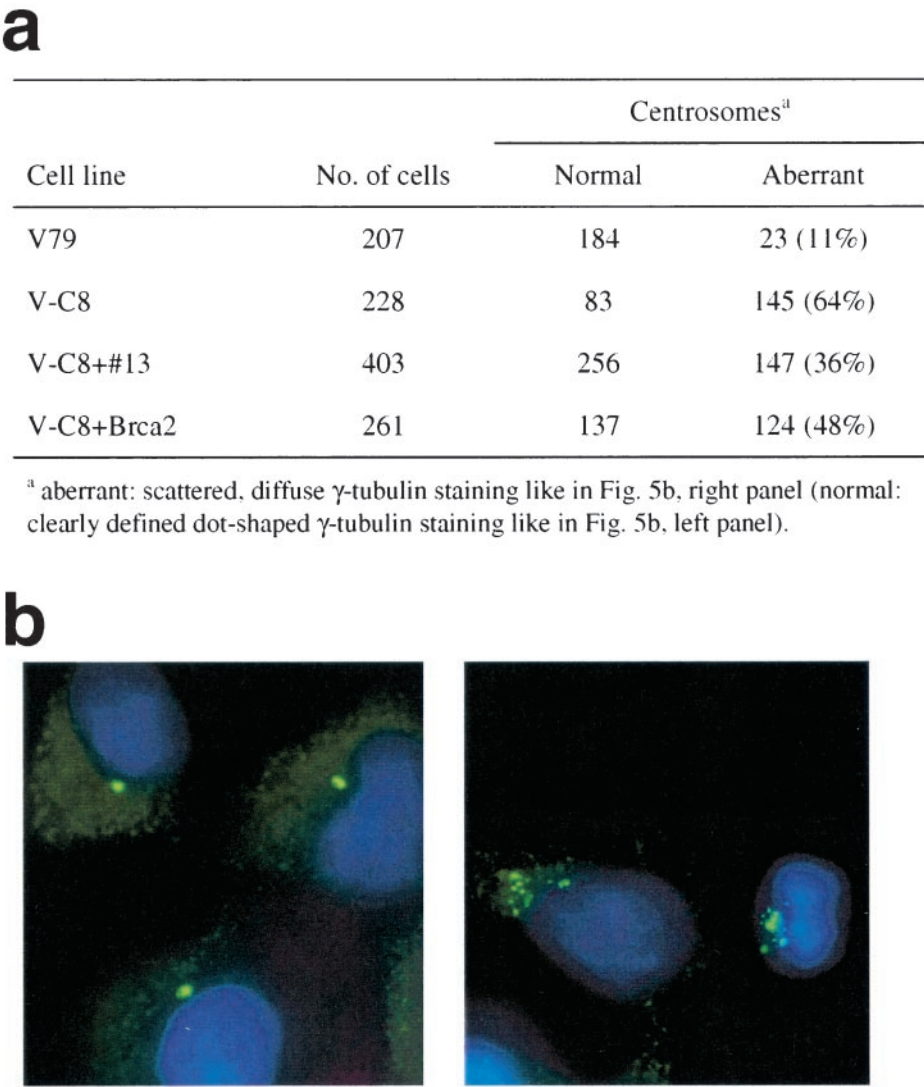
b

FIG. 5. (a) Centrosome analysis in interphase cells of wild-type V79, Brca2-deficient V-C8, V-C8 with human chromosome 13 providing the *BRCA2* gene (V-C8+#13, three independent clones), and V-C8 containing a BAC with the murine *Brca2* gene (V-C8+Brca2, two independent clones). (b) Immunofluorescent visualization of normal centrosomes in V79 cells (left panel) and abnormally structured centrosomes in V-C8 cells (right panel).

genomic integrity (35). How this protein performs these cellular functions remains largely unknown. The human *BRCA2* gene has 27 exons, encoding a protein of 3,418 amino acids. The large central exon 11 contains eight copies of 30- to 80-amino-acid BRC repeats, which are conserved across mammalian Brca2 proteins (2). Through these repeats Brca2 interacts with Rad51 (38). An additional Rad51-binding domain has been identified in the C-terminal region of mouse *Brca2*, which is largely conserved in the human sequence (22). Defective Rad51 focus formation has been demonstrated in the BRCA2-deficient human pancreatic adenocarcinoma cell line CAPAN-1, as well as in Brca2-deficient MEFs. Both of these cell lines encode Brca2 proteins truncated within exon 11, deleting the C-terminal Rad51 binding domain and part of the BRC repeats (44, 45).

To date, the majority of identified human germ line mutations in *BRCA2* are small deletions, which can be found along the whole protein (see the Human Gene Mutation Database [http:/www.uwcm.ac.uk/uwcm/mg/hgmd0.html]). Nearly all mutations have been predicted to result in a truncated BRCA2 protein, causing loss of the nuclear localization signals, possibly resulting in a defect in the subcellular localization of the protein (24). Besides aberrant subcellular localization of Brca2 itself, defects in Brca2 could affect the localization of Rad51, as we have found in V-C8 cells (Fig. 6). The amount of nuclear Rad51 is reduced in V-C8 compared to V79 cells. In accordance with this, it has been demonstrated in CAPAN-1 cells that Rad51 is localized mainly in the cytoplasm (4).

The interaction of BRCA2 with Rad51 (4, 38) and its effect on Rad51 localization (Fig. 6) (4) suggest that BRCA2 is specifically involved in DNA repair through HR. Indeed, by using the I-*Sce*I endonuclease to induce a DSB into a recombination repair substrate, which had been integrated in Brca2-deficient human CAPAN-1 or mouse embryonic stem (ES) cells, it has

TABLE 4. Types and locations of spontaneous mutations in the *hprt* gene of Brca2-deficient V-C8 cells

Mutation type	Mutant	Position[a]	Exon	Base change	Sequence change	Amino acid
Transversion	SP-17	617	9	GC→TA	ATTT(G)TGTC	Val→Phe
Double mutation	SP-7	530	7	AT→GC	CCAG(A)CTTT	Asp→Gly
		531	7	CG→AT	CAGA(C)TTTG	Asp→Glu
Insertion	SP-23	438–441	6	Insertion of 21 bp		
Frameshift	SP-5	156	3	Del. of 1 bp	GAGA(T)GTCA	
Deletion	SP-21	137	3	Del. of 8 bp		
	SP-11	Del. ex. 2	Del. ex. 2			
	SP-12	Del. ex. 4	Del. ex. 4			
	SP-14	Del. ex. 4	Del. ex. 4			
	SP-20	Del. ex. 2, 3, and 4	Del. ex. 2, 3, and 4			
	SP-2	No cDNA	Del. of entire gene			
	SP-8	No cDNA	Del. of entire gene			
	SP-13	No cDNA	Del. of entire gene			
	SP-15	No cDNA	Del. of entire gene			
	SP-16	No cDNA	Del. of entire gene			
	SP-19	No cDNA	Del. of entire gene			
Other	SP-3	No cDNA	All ex. present			
	SP-10	No cDNA	All ex. present			
	SP-22	No cDNA	All ex. present			
	SP-24	No cDNA	All ex. present			
	SP-25	Del. ex. 7	Splice			
	SP-18	No mutation found				

[a] Del., deletion; ex., exon(s).

been shown that Brca2 is essential for DSB repair via HR (18). Brca2 deficiency also leads to impaired spontaneous HR (18, 40), for which the interaction of BRCA2 with RAD51 appears to be necessary (40).

The large size of the BRCA2 protein suggests the existence of several additional functional domains able to interact with other proteins than Rad51, which act in various cellular processes (37). Consistent with this notion is the sensitivity of V-C8 cells toward a variety of DNA-damaging agents including, e.g., UV light and alkylating agents, which induce DNA lesions that are mostly not repaired by HR. Thus, BRCA2 is likely also involved in a general cellular response to DNA damage. Importantly, we found that V-C8 cells display RDS (Fig. 3) (36). RDS is a hallmark of cells derived from patients with AT, AT-like disorder, and Nijmegen breakage syndrome, which are defective in ATM, MRE11, and NBS1, respectively (21). Since the dose response of DNA synthesis inhibition in hamster cells differs from that in human cells, we used the AT-like hamster cell line V-E5 (47) to compare RDS levels. We found that the RDS level of V-C8 cells is similar to that seen in V-E5 cells and that the *BRCA2* gene corrects this phenotype (Fig. 3). Interestingly, it has been shown that the human BRCA1-deficient HCC1937 cells also display RDS (41). These results indicate that, in addition to ATM, MRE11, and NBS1, both BRCA1 and BRCA2 function in the S-phase checkpoint, giving a new view on this checkpoint, but exactly how the BRCA proteins function in this respect still needs to be elucidated.

V-C8 cells display enormous chromosomal instability (Tables 1 and 2) (19). Our data are in agreement with recent results showing that *BRCA2* deficiency leads to chromosome breakage and gross chromosomal rearrangements (44), as well as to centrosome abnormalities (34). Abnormal centrosomes, potentially giving rise to incorrect chromosome segregation, have also been found in *Brca1*-deficient MEFs (42) in the hamster cell mutants irs1 and irs1SF, which are defective in *Xrcc2* and *Xrcc3*, respectively (9), and in Mre11-deficient chicken cells (43), suggesting a link between DSB repair mechanisms and centrosome functioning, but the exact correlation is far from clear. Nevertheless, our results indicate that the centrosome abnormalities found in V-C8 cells are most probably not the main cause of the observed chromosomal instability, since only 1 of 18 cells with aberrant centrosomes had a multipolar spindle. The relatively poor complementation by either the human or the mouse *Brca2* gene could indicate differences between the Brca2 proteins of these species. However, this can be better addressed once the hamster cDNA is available.

We found a change in the spontaneous mutation spectrum of V-C8 cells compared to that of normal V79 cells. The majority of spontaneous mutations in V-C8 cells at the *hprt* locus are deletions (Table 4), while in wild-type V79 cells mainly base substitutions are found (49). This suggests that Brca2 is involved in a process preventing formation of deletions during replication. This phenotype is reminiscent of HR deficiency in *Rad54* knockout mouse ES cells, in which DSB repair through deletion formation is increased (5). The observation of chromosomal instability and the much higher frequency of spontaneous deletions provide a mechanistic basis for cancer development initiated by Brca2 deficiency.

The tremendous sensitivity of V-C8 cells toward cross-links indicates that Brca2 is important in protecting cells against these agents. Sensitivity to cross-links has also been found in
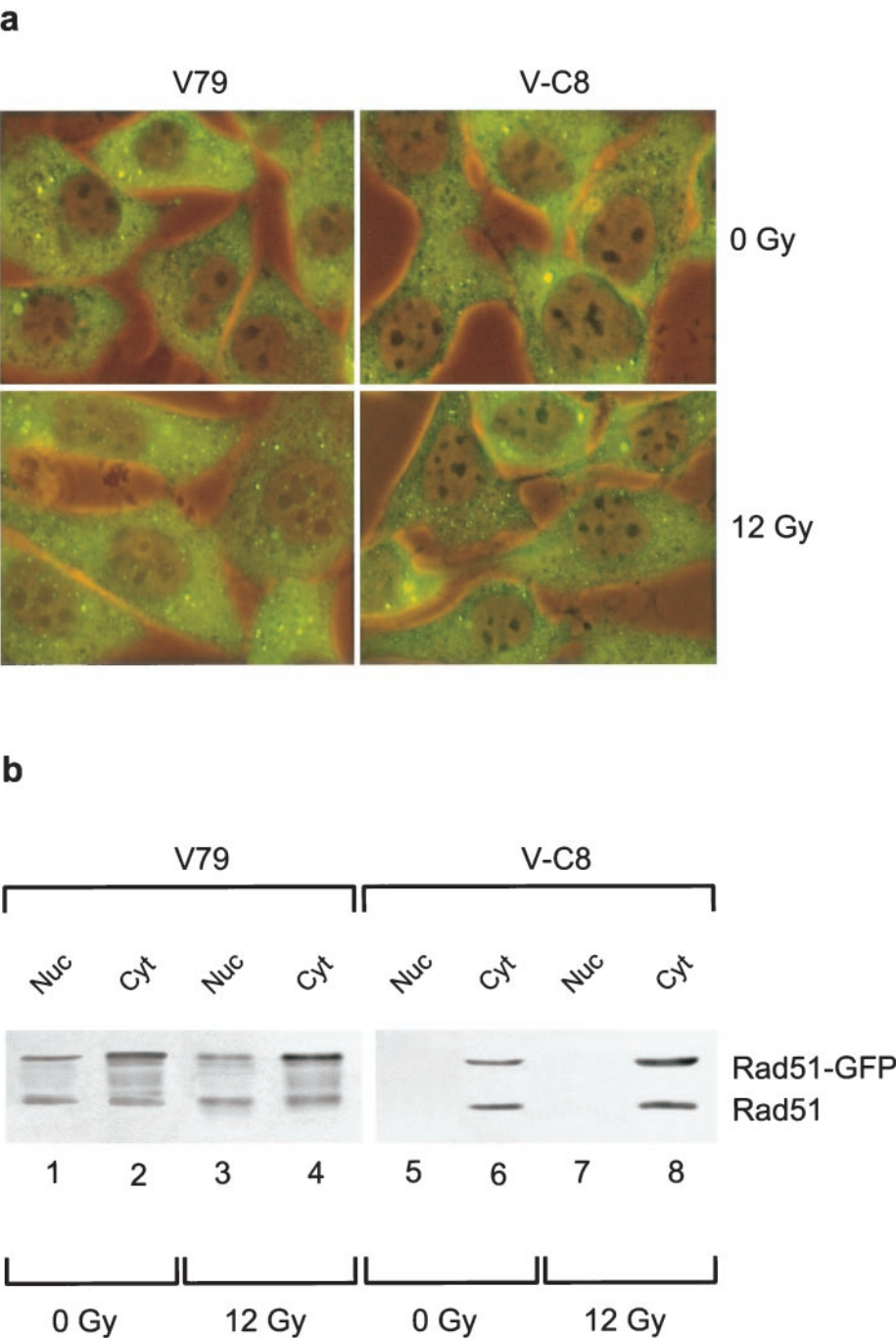
FIG. 6. Subcellular localization of Rad51-GFP in V-C8 cells. Stable transfectants of wild-type V79 or V-C8 cells expressing Rad51-GFP were analyzed by immunofluorescence (a) or immunoblotting with anti-Rad51 antibodies (b) at 6 h after mock treatment or irradiation with 12 Gy of gamma-irradiation.

Brca2-deficient mouse lymphocytes (44) and is displayed by hamster or chicken cell mutants defective in one of the Rad51 paralogs (16, 26) and by Brca1-deficient mouse ES cells (1, 17). In parallel, as in Brca2-deficient cells, defects in the above genes lead to chromosomal instability and impaired damage-induced Rad51 focus formation, suggesting a role for all of these proteins in maintaining genomic integrity through Rad51-dependent HR.

Our results indicate that hamster V-C8 cells offer a useful tool for studying the functions of the *Brca2* gene. Unlike the Brca2-deficient MEFs and CAPAN-1 cells, V-C8 cells grow well in culture, and large amounts of cells can be relatively

quickly obtained. In addition, they have a high cloning efficiency (25%) and can easily be transfected with exogenous DNA. The very high MMC sensitivity of V-C8 cells provides an easy readout in, for example, functional complementation studies. Currently, we cannot exclude the possibility that the extreme sensitivity of V-C8 cells to cross-links results from a specific mutation in *Brca2* in these cells. However, this finding might have important consequences for the therapy of some *BRCA2*-deficient breast cancer patients because they could be more responsive to treatment with cross-linking agents.

ACKNOWLEDGMENTS

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